TOBACCO INDUSTRY RESEARCH COMMITTEE
350 FIFTH AVENUE NEW YORK 1, N. Y.

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Application For Research Grant

Expendable Supplies
Permanent Equipment
Overhead
Other Travel

Date: Nov. 21, 1955

1. Name of Investigator: Victor Richards, M.D.

2. Title: Professor of Surgery. Executive Head of Dept. of Surgery

3. Institution & Address: Stanford University School of Medicine
Sacramento & Webster Sts., San Francisco 15, California

4. Project or Subject: A comparative study of the effects of whole and fractionated extracts of cigarette smoke and those of known carcinogens on lung and
I. The cytology and nuclear DNA content of epidermis in various strains of mice.
and/or II. The cytology and nuclear DNA content of lung and epithelium of the bronchial tree of mice and hamsters.

5. Detailed Plan of Procedure (Use reverse side if additional space is needed):

I. The cytology and nuclear DNA content of epidermis in various strains of mice. (This portion of the program would require at least three years if not longer).

EXPERIMENTS ON EPIDERMIS.

EXPERIMENT A. To test for carcinogenicity of cigarette smoke extract and the fractions thereof as compared to the effects of known carcinogens.

1. Materials.

a) Experimental animals - four different strains of inbred mice.

1. CAF_1 - the strain used by Wynder et al (Canc. Res. 13 (12) 855 (1953)). Since the efforts of British investigators along the same lines have given entirely negative results to date (Brit. Emp. Canc. Camp. 1954), it is felt that the work should be repeated with Wynder's strain using the extracts provided by the T.I.R.C.
2. C_3H (Strong) - used because tissue could be studied histologically and biochemically (DNA).
3. C_{57} black (Little)
4. I

b) Substances to be tested.

1. Whole condensate from composite cigarette smoke, to be supplied by the T.I.R.C.
2. Five fractions of this extract, AB, EA, J, K, M, - separated according to the method of Kosak (Proc. Amer. Assoc. Canc. Res. 1 (2) 27 (1954)). If the T.I.R.C. would prefer to prepare these fractions then we could obtain them from this source.
3. Known carcinogens a. 20 methylcholanthrene
b. 3:4 benzpyrene

Signature s/ Victor Richards, M.D.
Director of Project

s/ A. Y. Brandin
Business Officer of the Institution

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2. Method

a) Treat skins of mice with increasing strengths of whole extract of smoke, fractions thereof and known carcinogens, 20 methylcholanthrene and 3:4 benzpyrene

b) Controls would consist of
   mice painted with solvents alone
   untreated animals

c) Since according to Smith et al. (Proc. Amer. Assoc. Canc. Res. 1, 45 (1954)) early skin changes such as loss of hair within 10 days and nucleolar enlargement within 4 days correlate well with the carcinogenicity of compounds applied to the skins of mice a comparative study would be carried out on the
   histology
   cytology
   nuclear DNA content (microspectrophotometrically measured)
   during these early stages.

d) Similar studies would also be carried out at certain chosen later stages.

e) All other organs in the body would be examined at autopsy for evidences of tumor formation.

EXPERIMENT B. To test for the initiating action of cigarette smoke extract and fractions thereof.

1. Materials.

a) Experimental animals.
   The same four strains of mice as in experiment A.

b) Substances to be tested.
   The same whole condensates from composite smoke of cigarettes and fractions thereof as in experiment A.

2. Method

a) Make weekly applications of solutions of
   1. whole extract from cigarette smoke
   2. fractions of the above extract (especially fraction K, since positive results have been reported by Smith et al. (Proc. Amer. Assoc. Canc. Res. April 1954)) - followed by a known promoter such as croton oil.

   Groups of animals.
   a. In this group apply the whole extract in near maximal tolerated dose followed after an interval by repeated applications of croton oil.
   b. Do the same with each fraction of the extract, using a separate group of animals for each.
   c. Repeat a & b applying the test substances and croton oil alternately during part or all of the treatment period.
   d. Treat this group with croton oil alone.
   e. Controls treated with solvents alone.
   f. Untreated controls.

b) Compare results histologically, cytologically and microspectrophotometrically (DNA) in a, b, and c with those in d, e and f.

EXPERIMENT C. To test for promoting action of whole cigarette smoke extract and fractions thereof - CO-carcinogenesis.

1. Materials

a) Experimental animals. The same four inbred strains of mice as in A and B.

b) Substances to be tested. The same whole extract of cigarette smoke and fractions as in experiments A and B.

2. Method

a) After an initial treatment with a known initiator such as 3:4 benzpyrene make weekly applications of
   1. whole extract
   2. fractions of the extract

   Groups of animals.
   a. In this group an initial treatment with 3:4 benzpyrene will be followed with weekly applications of whole smoke extract.

Groups of animals (cont'd)

b. The same will be done here with each fraction of the extract, using separate groups for each.
c. 3:4 benzpyrene alone
d. controls treated with solvents alone
e. untreated controls.

b) Results in a, b, and c will be compared with those in d and e histologically, cytologically and microspectrophotometrically (DNA)

RESULTS IN EXPERIMENTS A, B, AND C WILL BE COMPARED.

The following experiments on lung and bronchial tree have been outlined as a possible alternative project in case the experiments on epidermis are too similar to projects being worked on by others. Or upon completion of project I this work could be continued for another three years.

II. The cytology and nuclear DNA content of lung and epithelium of the bronchial tree of mice and hamsters.

1. Materials.

a) Experimental animals.

1. Three strains of inbred mice with differing susceptibility to lung tumors.
   a. Strain A (Strong) extremely susceptible
   b. " C_3H " intermediate susceptibility
   c. " C_{57} black (Little) low "
2. Hamsters

b) Substances to be used.

1. Whole extract of cigarette smoke (supplied by the T.I.R.C.)
2. Fractions thereof-separated according to the method of Kosak (preferably supplied by the T.I.R.C.)
3. Known carcinogens-20 methylcholanthrene and 3:4 benzpyrene

Experiments with mice. A comparative histological, cytological and microspectrophotometric (DNA) study will be made of the effects of cigarette smoke extract, its fractions and of known carcinogens after introduction of these substances into the respiratory tracts of the three strains of mice using the two following methods

1. Method of T.D. Day (Brit. Emp. Canc. Camp. 1954). This is a modification of the method of Magnus (J. Path. & Bact. 49, 21 (1939)).
   Introduce the test substance (dissolved in a suitable solvent) on a blunt serum needle into the animal's mouth and then pass the tube into the esophagus and hold it there for a few seconds. This method was used by Day in preference to submitting the animals to aerosols of the tobacco tar as the latter method failed to result in effective concentrations in the lungs.
2. Method of Advervont. (U.S. Publ. Health Rep. 52, 1584 (1937).
   After anesthetizing the animal by the intraperitoneal injection of nembutal a fine thread saturated with the test substance is then drawn into the chest cavity by means of a fine needle passed between the ribs. The thread is then left within the lung. The mice are then sacrificed at chosen intervals and the lungs found to contain the threads (in some cases the lungs are missed) are studied as indicated above.
   (Andervont was able to produce not only adenomas and adenocarcinomas with this method but also squamous cell carcinomas - the type associated with cigarette smoking in man)
3. Controls in both methods would consist of animals treated similarly but without either smoke extract, its fractions or the carcinogens.

Experiments with hamsters. A comparative histological, cytological and microspectrophotometric (DNA) study will be made of the effects of whole extract of smoke, its fractions and of known carcinogens by directly instilling these substances into the left bronchus of hamsters.

permanent equipment

1 research microscope $2,000

1 microspectrophotometer complete with Leitz Panphot micros. U.V. light, Farrand photometer & monochromator $6,900

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1 full time technician - $3,600
1 animal caretaker 3,000
1 full time Ph.D. (cytologist & cytochemist) 6,000
1 part time secretary 1,500
$14,100

6. Budget Plan: Applied for Research Grant

	1 Yr.	3 Yrs.
Salaries	$14,100	$42,300
Expendable Supplies	2,000	6,000
Permanent Equipment	[illegible]	6,900
Overhead	1,000	3,000
Other Travel	900	2,700
Microspectrophotometric scanning attachment to be used interchangeably with Farrand photometer	XXX	5,000
	Total for 3 yrs	$65,900

Date Nov. 21, [illegible]

1. Name of Investigator: Victor [illegible] Title: Professor of Surgery, Executive Head of Dept. of Surgery

7. Anticipated Duration of Work: For project I - three years. If continued with project II - another 3 years.

8. Facilities and Staff Available: Institution: Stanford University School of Medicine

a. an entire laboratory to be used exclusively for this work in the dept. of surgery of the Stanford medical school

b. a small room on the same floor to be converted into an animal room

4. Project or Subject: A comparative study of the effects of whole and fractionated

Staff: consultative facilities with depts. of radiology, pharmacology and pathology cytology and nuclear DNA content of epidermis in various strains of mice.

9. Additional Requirements: Project II. The cytology and nuclear DNA content of lung and epithelium of the bronchial tree of mice and hamsters.

5. Detailed Plan of Procedure (Use reverse side if additional space is needed):

I. The cytology and nuclear DNA content of epidermis in various strains of [illegible]. (This portion of the program would require at least three years to complete).

10. Additional Information (including relation of work to other projects and other sources of supply):

a. Relation of work to other projects. The work could be related to a project to be launched in tissue culture in the dept. of surgery in which human lung tissue could be grown in hamster's cheek pouches. The direct effect of tobacco extracts and carcinogens on such transplants could be studied.

1. [illegible]

a) [illegible] If structural changes can be demonstrated in the lungs and bronchial tree, they could be correlated with physiological studies of pulmonary function and circulation [illegible] used by Wynder et al (Cancer Res. 13 (12) 855 (1953)). Since

b. Relation of work to other sources of supply. Fetal lung tissues could be obtained from this dept. (surgery) which could be cultivated in vitro (watch glass method) in the tissue culture lab on the same floor of the medical school. The direct effect of smoke extract on such tissue could be studied histologically

2. Cytologically and microspectrophotometrically (DNA).

3. C_{57} black (Little)

4. I

b) Substances to be tested.

1. Whole condensate from composite cigarette smoke, to be supplied by the T.I.R.C.

2. Five fractions of this extract, A1, EA, [illegible], F_4, F_5 - separated according to the method of Kosak (Proc. Amer. Assoc. Cancer Res. 1 12) 27 (1954)). If the T.I.R.C. would prefer to prepare these fractions that we could obtain them from this source.

3. Known carcinogens a. 20 methylcholanthrene

b. 3:4 benzpyrene

Signature s/ Victor Richards, M.D.
Director of Project

s/ A. F. Brandin
Business Officer of the Institution
Business Manager

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